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# Effect of Low Doses of Genistein and Equol on Protein Expression Profile in MCF-7 Cells

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**Abstract** Although action modes of equol and genistein have been extensively studied, their precise roles in tumor cells remain elusive. To address possible effects of these compounds on protein expression in mammary tumor cells, proteins modulated in MCF-7 mammary tumor cells when incubated in absence and presence of 10 uM equol or genistein were identified through 2-dimensional gel electrophoresis, MALDI-TOF MS/MS, and NCBInr database search using Mascot software. Most proteins differentially expressed in MCF-7 cells after treatment with 10 uM genistein or equol were identified as being the same. Exposure to both compounds caused decreased cellular expression of RNA-binding protein regulatory subunit and oncogene DJ1 tubulin beta-1 chain, and increased expression of heterogeneous ribonucleoproteins F and L, KH-type splicing regulatory protein, and translation elongation factor EF-Tu precursor. Genistein and equol at dose used in this study showed common action mechanism.

**Key words:** genistein, equol, proteomics, MCF-7 cell line

## Introduction

Compelling evidence from several epidemiological and animal studies support that consumption of dietary soy reduces cancer risk (1, 2). Soybeans have also been found to contain numerous biologically active compounds that may be important in breast cancer prevention (3), while soy isoflavones have attracted significant attention due to the growing experimental results that show modulation of genes related to the control of the cell cycle and apoptosis (4).

Isoflavonoids from legumes, such as genistein and daidzein, mainly exist in glycoside forms and are then hydrolyzed in the gut into aglycones that are easily absorbed into the intestinal epithelial cells (5). Soy isoflavones act as weak estrogens or anti-estrogens depending on their concentration in the medium. The biphasic effects of varying concentrations of genistein in a culture medium have already been reported in a number of *in vitro* studies (6). At a physiological dose of 0.1 to 1 uM, genistein stimulates cellular proliferation in MCF-7 cells (7). Genistein has also shown effects that are not normally associated with ER, including the ability to inhibit tyrosine kinase and DNA polymerase. In addition, other effects can take place at the cellular and biochemical levels, potentially influencing the metabolism of steroids and fatty acids, serum steroid carrier proteins (SHBG, alpha-fetoprotein), and the intracellular and transmembrane transfers of hormones to membranes and nuclear receptors. Genistein is also suspected to stimulate the growth of mammary carcinogen MNU-induced estrogen-dependent mammary tumors (8). In addition, there is also a possibility that estrogen receptor-positive breast cancer cells proliferate in response to genistein within a certain dose range and that, regardless of estrogen receptors, mammary tumor cells pre-exposed to isoflavones may develop resistance to chemotheraphy via the promoted expression of anti-apoptotic proteins.

In general, equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman) is not present at more than trace levels in the urine of most healthy adults; however, consumption of soy results in the production of equol from daidzein via the intestinal bacterial metabolism (9). Data from several clinical studies of hormone-dependent diseases indicate that equol, an isoflavone metabolite, may be an important constituent in the action and effectiveness of soy (10, 11, 12). Accordingly, because gensitein and equol have both been found to exhibit estrogen-like activities, the present study investigated whether these isoflavones exert their biological effects via a similar signaling pathway and regulate the expression of common proteins. Thus, the protein expression profile regulated by the two isoflavones was investigated in MCF-7 mammary tumor cells using a proteomic approach.

## Materials and Methods

**Reagents** Reagents used for gel electrophoresis and IPG strips (17 cm, pH 3-10, ReadyStrip™ IPG Strip) were purchased from Bio-Rad (Hercules, CA, USA), while the other reagents were obtained from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Trypsin used for in-gel digestion was purchased from Promega (Madison, WI, USA), and α-cyano-4-hydroxy-trans-cinnamic acid

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(CHCA) and other reagents used for the MALDI-TOF analysis were obtained from Sigma or Merck. Water was purified using a Milli-Q water purification system with a resistance of 18.2 Ohm. Eagle's Minimum Essential Media (EMEM) were obtained from Gibco-BRL (Grand Island, NY, USA), fetal bovine serum (FBS) purchased from Hyclone (Logan, UT, USA). Urea, thiourea, CHAPS, DTT, iodoacetamide, Trisma-Base, SDS, and glycerol were of electrophoresis grade.

**Cell culture** The human breast cancer MCF-7 cell line obtained from the Korean Cell Line Bank (Seoul, Korea) were cultured in EMEM containing 10% FBS and grown at 37°C under a 5% $CO_2$–95% air atmosphere. Confluent cells in 145-$cm^2$ plates (Nunc, Rochester, NY, USA) were washed twice with 15 ml phosphate buffer saline solution (PBS) and detached by scraping with a rubber policeman using 7 ml ice-cold PBS. The collected cells were centrifuged at 15,000 g, 4°C, for 25 min and subjected to lysis with 10 ml of 1% Triton X-100. The cell pellet was then suspended by pipetting and incubated in ice for 1 hr. After incubation, the cell lysate was centrifuged at 4°C for 20 min, and the supernatant was collected in a centrifugal tube (50 ml). The protein was then precipitated by adding trichloroacetic acid at one tenth of the total volume. The pellets were then washed with 10 ml ethanol twice, followed by washing with 1.2 ml ethanol for the 3rd wash, and 0.5 ml ethanol for the 4th and 5th washes. After washing, each pellet was dried in a Speed-Vac for 40 min, and the protein pellets were redissolved in a re-hydration buffer II (8~9.8 M Urea, 1~4% CHAPS, 15~100 mM DTT, 0.001% bromophenol blue). Protein concentration in the final supernatant was determined using a protein assay dye reagent (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. The sample was then stored in microfuge tubes at -70°C.

**Cell proliferation assay** For the growth study, MCF-7 cells were seeded in 96-well plates ($4 \times 10^3$ cells/well) in EMEM supplemented with 1% charcoal-stripped FBS for 24 hr, and treated with phytoestrogens (genistein, daidzein, equol) and 0.1 nM estradiol for 7 days. The medium was changed every 2 days. As an indirect measure of growth, sulforhodamine B (SRB) assay was used as described previously (13). Briefly, the medium was removed, washed with PBS, and the cells fixed by incubating with 10% TCA for 1 hr at 4°C. The plates were washed with tap water and dried. The cells were then stained with a 0.4 % SRB solution for 30 min. After washing the wells with 1% acetic acid, 10 mM Tris buffer (pH10.5) was added, and the signals detected by a microplate reader (model 550, BioRad) at 490 nm.

**Two-dimensional gel electrophoresis** Prior to the first dimension of gel electrophoresis, the samples were desalted using micro Bio-Spin P-6 columns containing 10 mM Tris–HCl buffer (BioRad) following the manufacturer's instructions. The desalted samples containing about 1.5 mg cytosolic proteins were dried using a vacuum centrifuge and redissolved in 300 ml rehydration buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS (w/v), 50 mM DTT, and 0.5% IPG buffer 3-10. The dry IPG strips (17 cm, pH 3–10) were then rehydrated for 13-14 hr with 400 μl sample in a focusing tray under a layer of mineral oil. The rehydrated strips were then run at 22°C with voltages increased stepwise as follows: 50 V for 13-14 hr, 10,000 V for 4 hr, with total Vh reaching 70,000 at the end of the run. The focused strips were then equilibrated for 10 min in equilibrium solution I (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 130 mM DTT) and equilibrium solution II (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 135 mM DTT). For the second dimension gel electrophoresis, the equilibrated strips were aligned on top of an SDS precast stacking gel (4% Tris-HCl gels, pH 6.8) and separating gel (12% Tris–HCl gels, 17×17 $cm^2$), and sealed with 1% molten agarose prepared in an SDS-PAGE running buffer containing trace amount of bromophenol blue. The protein separation was performed in a Protean II xi Cell (BioRad) at 9 mA/gel overnight at 15°C. At the end of the run, the gels were removed and fixed for 30 min in a solution containing methanol/water/acetic acid (45:50:5, v/v/v) followed by washes with deionized water five times for 3 min per each wash. The staining was then performed with 0.1% Coomassie blue R-250 in 40% methanol and 10% acetic acid for 2 hr at room temperature, followed by ample rinsing with a solution containing 40% methanol and 10% acetic acid until the desired contrast was achieved.

**Analysis of gel images** The stained gels were scanned using an Epson GT-9500 scanner, and the images exported to the image analysis software program, PDQuest (Bio-Rad). While some spots over a certain level of intensity were automatically detected, other spots were detected manually (14). The intensities of the spots on the gel were normalized and compared with an imaginary standard created by merging the gel images being compared.

**Identification of gel-separated proteins** The protein spots were excised from the gels, sliced to a thickness of about 1 mm, and transferred to a microfuge tube containing 600 μl deionized water. The washed gel slices were then broken into fine particles with a pestle, incubated for 20 min in a wash solution (50% ACN in 50 mM $NH_4HCO_3$) to remove the staining dye, and dried in a vacuum centrifuge for 60 min. Subsequently, 20 μl trypsin solution (10 ng/μl in 25 mM $NH_4HCO_3$) was added to the tubes containing the gel particles, and the mixtures incubated at 37°C for 16-20 hr. Following digestion, the peptides were extracted in a buffer (5% TFA in 60% ACN), concentrated by Speed-Vac centrifugation for 4 hr, resolubilized in a resuspension solution (0.1% TFA in 50% ACN), sonicated in a bath for 5 min, and centrifuged for 2 min. Tryptic peptide solutions were mixted at a 1:1 ratio with 10 mg/ml CHCA matrix in 0.3% TFA, and spotted on stainless steel MALDI smaple plates. Peptitde mixtures were then analyzed using MALDI/TOF/TOF (4700 Proteomics Analyzer, Applied Biosystems, Framingham, MA, USA). Bradykinin, angiotensin I, and neurotensin were used as the internal and/or external calibrants for the spectra calibration. The spectra were acquired in the delayed extraction-reflector mode and standard conditions of 20,000 V acceleration voltage and 150 ns delay time. The peptides were selected within a mass range of 700-

3500 Da, while the spectra obtained by averaging 300 individual laser shots were calibrated internally and/or externally with the calibrants and trypsin autolytic products at m/z 842.5, 1045.6, and 2211.1. Typically, doubly and sometimes triply charged ions were selected for the MS/MS analysis (14). Protein identification was performed with an interfaced script of the Mascot search engine (http://www.matrixscience.com) that used the raw MS/MS data to search the NCBI protein database. In general, protein identification is considered accurate when the MS/MS results of three or more peptides in a given sample identify the same protein.

## Results

**Change of protein expression pattern in MCF-7 cells exposed to genistein** Steady-state level of the protein spectrum changed after exposing the cells to 10 uM genistein for 24 hr. More than 500 protein spots were resolved per 2D-gel from the total cell lysate (Fig. 1). Ten proteins, whose expressions either increased or decreased after genistein treatment in the MCF-7 cells, were identified through MALDI-TOF analysis. In the MCF-7 cells exposed to 10 uM genistein, the expressions of the RNA-binding protein regulatory subunit (oncogene DJ1) and tubulin beta-1 chain decreased, while those of the heterogeneous ribonucleoproteins F and L, KH-type splicing regulatory protein, translation elongation factor EF-Tu precursor, heat shock 70 kD protein 9B (mortalin-2), Cu/Zn superoxide dismutase, and prosomal protein p30-33k were elevated (Table 1).

**Change of protein expression pattern in MCF-7 cells exposed to equol** In the MCF-7 cells exposed to 10 uM equol, proteins showing decreased expression by more than 5-fold included the oxygen-regulated protein precursor, tubulin beta-1 chain, reticulocalbin 1 precursor, and RNA-binding protein regulatory subunit (oncogene DJ1) (Fig. 2, Table 2). On the other hand, in MCF-7 cells treated with 10 uM equol, expressions of heterogeneous ribonucleoproteins L & F, nucleophosmin-anaplastic lymphoma kinase fusion protein, translation elongation factor EF-Tu precursor, Lasp-1 protein, coproporphyrinogen oxidase (EC 1.3.3,3) precursor, mitochondrial KH-type splicing regulatory protein, mitochondrial outer membrane protein (TOM40), paraspeckle protein 1, and triosephosphate isomerase increased (Fig. 2, Table 2).

## Discussion

Although soy isoflavones have been recognized as promising cancer preventive and/or therapeutic agents for various cancers, their action mechanisms remain elusive. In particular, they have a biphasic effect on ER-positive cells depending on their concentration in the culture media, while inhibit estrogen action at high concentrations and exhibit estrogen-like activity at low concentrations. Furthermore, soy isoflavones have been reported to promote the proliferation of ER-positive cells at a physiologically relevant dose range. Thus, the cancer preventive effect of soy isoflavones may be associated with the down-regulation of the ER-alpha and ER-mediated signaling

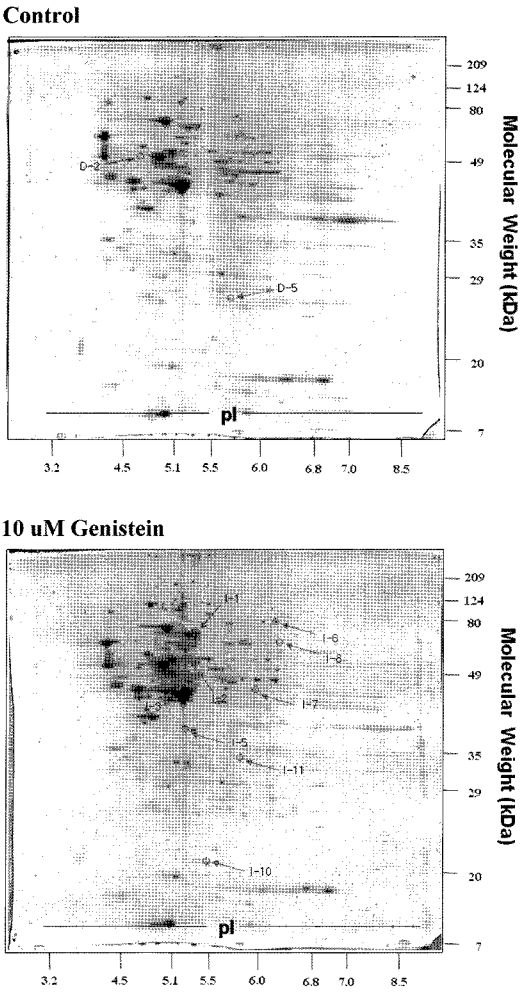



**Fig. 1. Changes in protein expression pattern in MCF-7 cells after genistein treatment.** Proteins (~500 ug) from control cells (A) and cells treated with 10 uM genistein (B) for 24 hrs were separated by isoelectrofocusing (IEF) and SDS-PAGE, followed by staining with Coomassie brilliant Blue R-250. The 2-D analysis was performed in triplicate with similar results. D-2, 5 represent protein spots whose intensities decreased, while I-1~I-11 represent protein spots whose intensities increased.

pathway, which induces the differentiation of mammary cells when prepubertally exposed to such compounds (29).

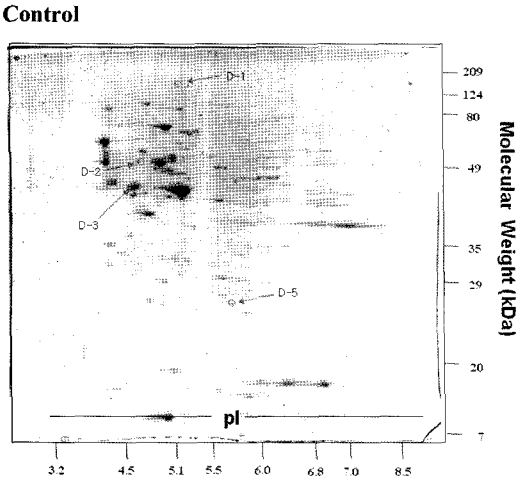
However, in this study, we were able to identify more than 10 types of proteins, whose expression levels were altered when treated with equol or genistein. The proteins induced by the phytoestrogens appeared to be involved in RNA processing, translation, and cell proliferation, which was consistent with the cell growth-stimulatory effect of the compounds at the dose used in this study (Fig. 3).

Tubulin-beta 1 and the RNA-binding protein regulatory subunit (oncogene DJ1) were both downregulated when treated with genistein or equol. Tubulin-beta 1 is the principal structural component of microtubules, which are important components of the surface membrane cytoskeleton and, together with the cytoplasmic and actin-rich cytoskeleton, responsible for the intracellular transport of vesicles, cell morphogenesis, and chromosome segregation during cell division in all eukaryotes.

Commonly, the upregulated proteins, including the

**Table 1. Identification of differentially expressed proteins from MCF-7 mammary tumor cells exposed to 10 uM genistein for 24 hrs**

| | Mascot score | Accession # | Protein name | Mr/pI | Cov (%) | Function |
|---|---|---|---|---|---|---|
| Decreased | 145 | gi135448 | Tubulin beta-1 chain | 49727/4.75 | 8 | constituent of microtubules (15) |
| | 105 | gi31543380 | RNA-binding protein regulatory subunit; oncogene DJ1 | 19878/6.33 | 69 | chaperone (16) |
| | 423 | gi12653415 | heat shock 70kD protein 9B (mortalin-2) | 73682/6.03 | 9 | chaperones, protein folding (17) |
| | 108 | gi4826760 | heterogeneous ribonucleoprotein F | 45643/5.38 | 4 | provide the substrate for the processing events that pre-mRNAs (18) |
| Increased | 162 | gi28336 | mutant beta-actin | 41786/5.22 | 7 | - |
| | 201 | gi4504865 | KH-type splicing regulatory protein | 73116/6.84 | 31 | mRNA trafficking (19) |
| | 443 | gi2136315 | translation elongation factor EF-Tu precursor | 49509/7.70 | 13 | protein biosynthesis (20) |
| | 74 | gi4557645 | heterogeneous ribonucleoprotein L | 60149/6.65 | 24 | provide the substrate for the processing events that pre-mRNAs (18) |
| | 234 | gi408239 | Cu, Zn superoxide dismutase, SOD= SOD1 gene product | 15823/5.70 | 24 | destroys radicals |
| | 79 | gi190447 | prosomal protein p30-33k | 30208/6.51 | 11 | ATP-dependent proteolytic activity (21) |



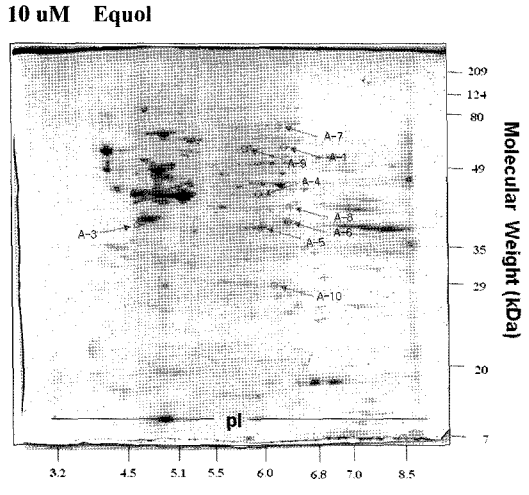




**Fig. 2. Changes in protein expression pattern in MCF-7 cells after equol treatment.** Proteins (1.5 mg) from control cells (A) and cells treated with 10 uM equol (B) for 24 hrs were separated by isoelectrofocusing (IEF) and SDS-PAGE, followed by staining with Coomassie brilliant Blue R-250. The 2-D analysis was performed in triplicate with similar results. D-1~D-5 represent protein spots whose intensities decreased, while A-1~A-10 represent protein spots whose intensities increased.

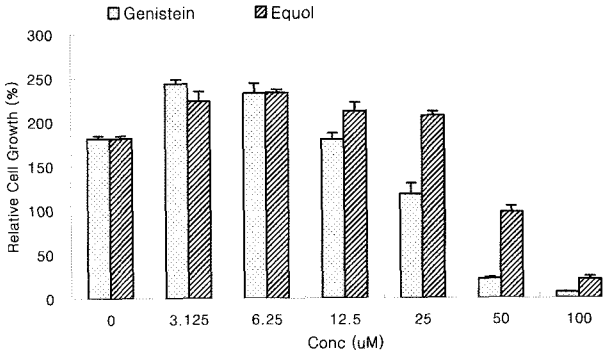



**Fig. 3. Dose-response effect of phytoestrogens on MCF-7 cell growth.** The MCF-7 cells (4,000 cells/well) in 96-well plate were cultured in the absence and presence of various concentrations of genistein, daidzein, and equol for 72 hrs, followed by an SRB assay.

heterogeneous ribonucleoproteins L & F, translation elongation factor EF-Tu precursor, and mitochondrial KH-type splicing regulatory protein, are associated with increased protein synthesis, which may be responsible for enhanced cell growth. A recent study showed that heterogeneous nuclear ribonucleoprotein F/H proteins were involved in the alternative splicing of the apoptotic mediator Bcl-x (30). However, the present study did not find any induction of BRCA1, a tumor suppressor gene that participates in DNA damage repair processes, as observed in another study (29).

Equol is the end-product of the biotransformation of the phytoestrogen daidzein, one of the two main isoflavones found in abundance in soybeans and most soy foods. Once formed, equol is a relatively stable estrogenic compound, having an affinity for both estrogen receptors, ER-alpha and ER-beta. However, equol is not produced in all healthy adults in response to dietary soy or daidzein, but is exclusively a product of the intestinal bacterial metabolism of dietary isoflavones (9). Several recent dietary intervention studies examining the health effects of soy isoflavones have suggested the potential importance of equol based on evidence that the maximal clinical responses to soy protein

**Table 2. Identification of differentially expressed proteins from MCF-7 mammary tumor cells exposed to 10 uM equol for 24 hrs**

| | Mascot score | Accession # | Protein name | Mr/pI | Cov (%) | Function |
|---|---|---|---|---|---|---|
| | 128 | gi5453832 | oxygen regulated protein precursor | 111266/5.16 | 19 | Cytoprotective cellular mechanisms (22) |
| | 145 | gi135448 | Tubulin beta-1 chain | 49727/4.75 | 8 | constituent of microtubules (15) |
| Decreased | 336 | gi4506455 | reticulocalbin 1 precursor | 38866/4.86 | 22 | regulate calcium-dependent activities (23) |
| | 105 | gi31543380 | RNA-binding protein regulatory subunit; oncogene DJ1 | 19878/6.33 | 69 | chaperone (18) |
| | 74 | gi4557645 | heterogeneous ribonucleoprotein L | 60149/6.65 | 24 | provide the substrate for the processing events that pre-mRNAs (18) |
| | 108 | gi4826760 | heterogeneous ribonucleoprotein F | 45643/5.38 | 4 | provide the substrate for the processing events that pre-mRNAs (18) |
| | 124 | gi609342 | nucleophosmin-anaplastic lymphoma kinase fusion protein | 75266/6.44 | 5 | assembly and/or transport of ribosome (24) |
| | 443 | gi2136315 | translation elongation factor EF-Tu precursor | 49509/7.70 | 13 | protein biosynthesis (20) |
| Increased | 158 | gi2135552 | Lasp-1 protein | 29786/6.11 | 14 | amplified in breast cancer,metastatic axillary lymph nodes (25) |
| | 323 | gi547615 | coproporphyrinogen oxidase (EC 1.3.3,3) precursor, mitochondrial | 40277/6.68 | 16 | Heme biosynthesis (26) (Cooper et al., 2005) |
| | 201 | gi4504865 | KH-type splicing regulatory ptotein | 68393/8.52 | 31 | mRNA trafficking (19) |
| | 223 | gi5174723 | mitochondrial outer membrane protein TOM40 | 37869/6.79 | 17 | import of protein precursors into the mitochondria (27) |
| | 64 | gi8922789 | paraspeckle protein 1 | 41713/5.93 | 3 | mRNA processing (28) |
| | 417 | gi999892 | chain A, triosephosphate isomerase (Tim) | 26522/6.51 | 26 | glycolysis |

diets were observed in equol-producers. Although equol has been reported to exhibit several biological activities, such as antioxidant activity, its precise action mechanism in cancer prevention is still unclear. However, in the present study, equol at low doses was found to regulate the protein expression level in MCF-7 cells with a similar pattern to genistein, suggesting that the biological functions of equol may operate through a pathway similar to that of genistein.

Phytoestrogens exert biological activity via nongenomic pathways as well as genomic pathways (30-31). In particular, genistein, a well-known tyrosine kinase inhibitor, can cause a variety of cellular events by modulating the phosphoryation of proteins associated with the cell cycle, cellular differentiation, and proliferation. Genistein has already been shown to inhibit the activation of NF-kappa B and Akt signaling pathways, both of which maintain a homeostatic balance between cell survival and apoptosis and are involved in the control of cell growth, apoptosis, inflammation, stress response, and many other physiological processes (4). Nonetheless, the cellular action of phytoestrogens appears to be dose-dependent. At a dose lower than 25 uM, equol and genistein both stimulated cell proliferation, while inhibited MCF-7 cell growth at a dose higher than 100 uM (Fig. 3). In general, at high doses, genistein and equol inhibited the growth and survival of the MCF-7 cells, most likely by inhibiting the intrinsic tyrosine kinase activities of the growth factor receptors or interfering with the cell signaling pathway. However, at low concentrations (<20 uM), genistein was found to mimic the action of 17-β-estradiol (E2) and stimulate the cell proliferation through its interaction with ER and inducement of E2-dependent gene expression (32); the relatively low phytoestrogen concentration used in this study may have been within the range at which promotion of the cell growth occurs via interaction with ER, thereby inducing the estrogen-dependent gene expression, explaining the similar protein expression profile observed. Therefore, the similar protein expression patterns induced by both equol and genistein at the dose used in this study indicate a shared action mechanism, at least in ER-positive MCF-7 cells. However, further studies are required to determine how the proteins regulated by equol and genistein treatments are linked to cancer preventive action *in vivo*.

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